

## A Modified Method for the Estimation of Acetylisoniazid in Urine

G. Raghupati Sarma, Chandra Immanuel, S. Kailasam, M. Kannapiran, N.G.K. Nair and S. Radhakrishna

*Tuberculosis Chemotherapy Centre, Madras-600031.*

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The method of Venkataraman et al. (1968) for the direct estimation of acetylisoniazid in urine has been modified to make it suitable for application to urine specimens containing sugar. The urine is first extracted with a mixture of chloroform and n-butanol, the organic phase re-extracted with dilute sulphuric acid, and the original method then applied to the acid extract. With the modified method, recoveries of acetylisoniazid were quantitative, both in the presence and the absence of glucose. Interference due to hydrazones and isonicotinylic acid was substantially reduced by the modification, while that due to isoniazid remained the same. There was little or no interference from isonicotinic acid, with either the original or the modified method.

### Introduction

With the advent of intermittent regimens in the chemotherapy of tuberculosis, the rate of inactivation of isoniazid (INH) has attained considerable prognostic importance (Tuberculosis Chemotherapy Centre, Madras, 1970; 1973). Simple methods for classifying subjects as slow or rapid inactivators of isoniazid, based on the ratio of urinary excretion of acetylisoniazid (Ac. INH) to INH, have been described (Eidus **et al.**, 1971; Venkataraman **et al.**, 1972; Ellard **et al.**, 1973a, b). While there are several methods for estimating INH in urine, the only known direct methods for estimating Ac. INH in urine are those described by Venkataraman et al. (1968) and Ellard **et al.** (1972).

In the course of routine estimations of Ac. INH at this Centre by the direct method of Venkataraman **et al.** (1968), it was noticed that a few urine specimens produced a yellow colour instead of the expected pink colour. Preliminary investigations showed that these specimens had been collected from diabetic patients, and that the presence of glucose ( $\geq 2.5$  mg./ml.) in urine significantly interfered with the estimation of Ac. INH. To resolve this difficulty, it was decided to first obtain an acid extract from urine (as for the estimation of free INH), and then employ the direct method of Venkataraman **et al.** (1968). The findings with the modified method (extraction method), both in the presence and the absence of glucose, are described in this paper; information on possible interference from other substances such as INH, isonicotinic acid (INA), isonicotinylic acid (INAG), monoacetylhydrazine (MAH), diacetylhydrazine (DAH), acid labile pyruvic acid isonicotinylic hydrazone (H-INH-PA) and

$\alpha$  -ketoglutaric acid isonicotinyl hydrazone (H-INH-KA) is also presented for this method, as well as for the direct method.

### Material and Methods

Pure INH and INA were purchased from Bayer & Co. (West Germany) and L. Light & Co. (U.K.), respectively. Ac.INH (Fox and Gibas, 1953), H-INH-KA (Dymond and Russell, 1970), and DAH (Turner, 1947) were synthesized as described. MAH was prepared employing a general method for the preparation of acid hydrazides (Vogel, 1971). H-INH-PA and INAG were gifts from Dr. G.A. Ellard. All the other chemicals used were of analytical grade.

**Direct method :** The method described by Venkataraman *et al.* (1968), without prior oxidation using potassium permanganate and without the reverse blank procedure, was employed; however, with a view to extend the range of estimation, 0.6 ml. of urine diluted to 1.0 ml. with water was used, instead of 1.0 ml. of urine.

**Extraction method :** The method of Rao *et al.* (1971) was employed for obtaining an acid extract from urine. Thus, to 3 ml. of urine, 1 ml. of 0.1N sodium hydroxide was added, followed by 3.2 g. of ammonium sulphate and 30 ml. of a mixture of chloroform and n-butanol (7 : 3). The contents were shaken on a rotary shaker for 30 min. (the organic extract was clarified, if necessary, with the addition of more ammonium sulphate), and filtered through cotton wool. Next, 20 ml. of this extract were shaken with 2.0 ml. of 0.1N sulphuric acid for 15 min. 0.4 ml. of 0.1 N sodium hydroxide was added to 0.6 ml. of the acid extract, giving a pH of  $5.8 \pm 0.2$ ; the Ac. INH content was then estimated by the direct method.

For routine classification of patients as slow or rapid inactivators of isoniazid a larger volume of this extract was required since both Ac. INH and INH had to be estimated. Consequently, some modifications were introduced in the above procedure. Thus, to 5 ml. of urine, 1.7 ml. of 0.1N sodium hydroxide were added, followed by 5.2 g. of ammonium sulphate and 30 ml. of a mixture of chloroform and n-butanol (7 : 3). The contents were shaken on a rotary shaker for 30 min. Next, 20 ml. of this extract (clarified, if necessary) were shaken with 6.0 ml. of 0.1N sulphuric acid for 15 min. and the Ac.INH content determined as described in the above paragraph.

### Results

**Recovery of acetylisoniazid and interference due to glucose :** On each of 10 days, Ac. INH was set up in concentrations of 0, 5, 10, 20, 30, 40, 50 and 60  $\mu\text{g./ml.}$  in normal urine (from volunteers), and in urine containing a high concentration of glucose (50 mg./ml.) After randomization, the specimens were processed by both the extraction and direct methods and the optical densities determined. Water standards were also set up in parallel. The geometric means are presented in Table 1.

With Ac. INH concentrations of 5 to 60  $\mu\text{g./ml.}$  the findings with the extraction and direct methods were similar in normal urine, and closely resembled those with the

Table I. Optical density readings by direct and extraction methods.

		Mean optical density* with the following concentration of acetylisoniazid ( $\mu\text{g./ml.}$ )						Mean optical density with blanks	
		5	10	20	30	40	50		60
<b>Water standard</b>		0.091	0.179	0.359	0.531	0.708	0.919	1.096	0.030
<b>Normal urine from volunteers</b>	Extraction method	0.087	0.173	0.356	0.526	0.716	0.914	1.081	0.032
	Direct method	0.084	0.172	0.352	0.533	0.711	0.906	1.091	0.070
<b>Urine containing glucose 50 mg./ml.</b>	Extraction method	0.085	0.179	0.355	0.532	0.716	0.895	1.074	0.031

\*After deducting values

water standards, indicating that the recovery of Ac. INH was quantitative by both the methods. With blank specimens the mean optical density with the extraction method was very similar to that with the water standard, but that with the direct method was considerably higher.

The presence of glucose (50 mg./ml.) did not affect the findings with the extraction method. However, with the direct method, the blanks had a mean optical density of 0.250 in the presence of glucose as compared with 0.070 in the absence of glucose ( $P < 10^{-4}$ ). Further, the mean optical densities (unadjusted for blanks) in the presence of glucose were 0.258, 0.266, 0.296, 0.315, 0.330, 0.364 and 0.390, respectively, for Ac. INH concentrations of 5, 10, 20, 30, 40, 50 and 60  $\mu\text{g./ml.}$ ; the corresponding values in the absence of glucose were 0.154, 0.242, 0.422, 0.603, 0.781, 0.976 and 1.161, respectively. Thus, the presence of glucose resulted in a failure of the direct method; this failure persisted even when the reverse blank procedure of Venkataraman *et al.* (1968) was employed (unpublished data).

**Sensitivity :** Blank urine specimens from 59 patients were processed by the extraction method; all the specimens had yielded a negative result for Ac. INH by the qualitative test of Eidus and Hamilton (1964). The geometric mean optical density for the water blanks set up in parallel was 0.034. The corresponding mean for the blank urine specimens was 0.035 and the 99 per cent confidence limits were 0.018 and 0.070. The upper limit corresponds to a concentration of 2  $\mu\text{g./ml.}$ , which may therefore be regarded as the sensitivity of the test.

**Reproducibility :** On each of 3 days, Ac. INH was set up in concentrations of 0, 5, 10, 20, 40 and 60  $\mu\text{g./ml.}$  in normal urine from volunteers. Four aliquots of each concentration were made, all the aliquots randomized and processed by the extraction method, and the optical densities determined. Beer's law was followed in this range of concentrations, the correlation between concentration and mean optical density being 0.9997. The coefficient of variation for replicate estimations was 4-6 per cent for concentrations of 5-10  $\mu\text{g./ml.}$ , and 2-3 per cent for concentrations of 20-60  $\mu\text{g./ml.}$

**Interference due to various compounds :** On each of 5 days (4 only in the case of INH), known concentrations of Ac. INH were added to aliquots of normal urine (from volun-

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teers), together with known concentrations of one of several other metabolites of isoniazid (Table II). The aliquots were randomized, processed by both the direct and extraction methods, and the optical densities determined. Next, the differences between the actual Ac. INH concentrations added and the observed Ac. INH concentrations in the presence of different concentrations of each compound were obtained. A pooled regression analysis of 'the difference' on 'the concentration of the compound' was then undertaken, to provide an estimate of the proportion of the compound that was estimated as Ac. INH. This proportion has been termed as 'Interference' due to the compound, and has also been expressed on a molar basis (Table II).

Table II. Interference due to various compounds in the estimation of acetylisoniazid by direct&d extraction methods.

Concentrations of Ac. INH ( $\mu\text{g}/\text{ml}$ )	Compound and concentrations ( $\mu\text{g}/\text{ml}$ )	Method of estimating Ac. INH	Interference*	Interference, expressed on a molar basis
0, 20, 60	INH 0, 10, 20, 40, 80, 160, 320	Direct Extraction	7.3% 6.2%	5.6% 4.7%
0, 10, 20, 40	{ H-INH-KA** 0, 10, 20, 40 H-INH-PA** 0, 10, 20, 40 INAG++ 0, 10, 20, 40	Direct	56%	43%
		Extraction	6.6%	5.1%
		Direct	41%	31%
		Extraction	Not tested	—
0, 20, 40, 60	INA 0, 10, 20, 40, 60, 80	Direct Extraction	1.1% Nil	0.8% Nil
0, 10, 20	MAH/DAH 0, 5, 10, 20	Direct Extraction	Nil Nil	Nil Nil

\* Expressed as the percentage of the compound that is estimated as Ac. INH.

\*\* Concentrations expressed as INH equivalent.

++ Concentrations expressed as INA equivalent.

The interference due to INH was 7.3 per cent by the direct method and 6.2 per cent by the extraction method. Considering next the interference due to hydrazones, the presence of H-INH-KA or H-INH-PA, even in a concentration of 10  $\mu\text{g}/\text{ml}$ . (INH equivalent), resulted in significant overestimation of the Ac. INH content by the direct method. It was computed that 56 per cent of H-INH-KA and 41 per cent of H-INH-PA were estimated as Ac. INH by this method. With the extraction method, the interference from the hydrazones might be expected to be considerably less because they would be converted to INH, the interference due to which is relatively small. In the event, an investigation with H-INH-KA showed that the interference was 6.6 per cent (H-INH-PA could not be investigated due to paucity of the compound).

The interference due to INAG was considerably less with the extraction method (3.8 per cent) than with the direct method (13 per cent). As regards INA, the inter-

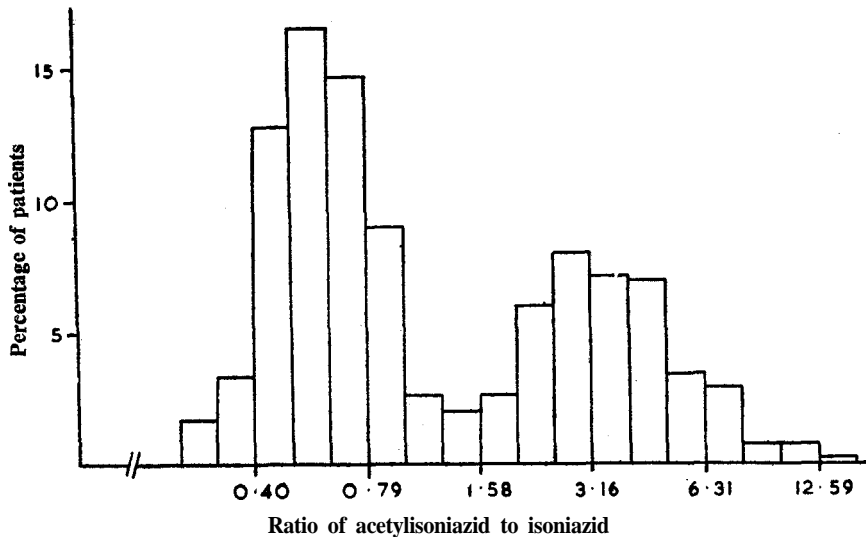
ference was slight with the direct method (1.1 per cent) and nil with the extraction method.

There was no interference from MAH or DAH, either with the direct or with the extraction method.

**Classification of patients as slow or rapid inactivators of isoniazid :** The ratio of Ac. INH to INH in urine collected over the period 3-4 h. following an intramuscular dose of INH 3 mg./kg. body-weight was determined for 458 patients. Ac. INH was estimated by the extraction method, and INH by the method of Rao *et al.* (1971). The graph illustrates the findings on a logarithmic scale, the logarithmic transformation having been employed to obtain approximately the same error variance (that is, the variance between duplicate estimations) for rapid and slow inactivators of isoniazid. The distribution is bimodal and suggests that the patients with a ratio of 1.25 or more are rapid inactivators and those with a ratio of 1.24 or less are slow inactivators. Employing this criterion 182 (40 per cent) of the 458 patients were classified as rapid, and 276 (60 per cent) as slow inactivators.

#### Graph

Distribution of 458 patients according to the ratio of acetylisoniazid to isoniazid in urine for the period 3-4 h. after an intramuscular dose of isoniazid 3 mg./kg. body-weight.



In a sub-sample of 142 consecutive patients, the Ac. INH was also estimated by the direct method, and the patients classified as rapid or slow inactivators, the criterion for a rapid inactivator being a ratio of 2.00 or more (Venkataraman *et al.*, 1972). In 141 of the 142 patients, the classification was identical by the extraction and direct methods. The lone exception had a ratio of 1.26 by the extraction method (rapid inactivator) and 1.63 by the direct method (slow inactivator); at 4½ h. after the intramuscular test dose, this patient's serum INH concentration, estimated by the

spectrophotometric method of Rao **et al.** (1971), was 0.63  $\mu\text{g./ml.}$  (rapid inactivator). In the 141 patients with an identical classification by the two methods, the discrimination, assessed as the ratio of the difference in means between rapid and slow inactivators to its standard error, was slightly higher with the extraction method (26.0) than with the direct method (23.7).

### Discussion

Until the publication of Ellard **et al.** (1972), the only method available for the direct estimation of Ac. INH in urine was that of Venkataraman **et al.** (1968). Our observations have shown that the presence of glucose ( $\geq 2.5$  mg./ml.) in urine results in the development of a yellow colour with the latter method instead of the expected pink colour, this possibly being due to a reaction of glucose with the cyanogen chloride liberated. The presence of a very high concentration of ascorbic acid (10 mg./ml.) has also been found to result in a yellow colour (unpublished data). Obviously, in such circumstances, estimates of Ac. INH content by this method will not be accurate. With the extraction method described in this paper, there was no interference from either ascorbic acid (unpublished data) or glucose, because neither is extracted into the chloroform-butanol layer. The recoveries were quantitative both in the presence and absence of sugar, and Beer's law was followed over the range 5-60  $\mu\text{g./ml.}$  Finally, the blank readings were low and similar to those obtained with water, while those with the direct method were considerably higher.

Interference from INH is about the same with the direct and the extraction method, and is of the order of 6-7 per cent. As regards INA, there is little or no interference with either method. One method of reducing the interference due to INH, suggested by Venkataraman **et al.** (1968), is to oxidize the INH to INA by employing potassium permanganate. However, this reaction is highly sensitive, and it is difficult to determine the exact endpoint for the addition of potassium permanganate. Also, there is the possibility that long exposure to potassium permanganate may oxidize the Ac. INH also to INA.

The presence of hydrazones in urine leads to considerable over-estimation of Ac. INH by the direct method, as 56 per cent of H-INH-KA and 41 per cent of H-INH-PA are also estimated as Ac. INH. In contrast, the interference is slight (about 7 per cent) with the extraction method, as the hydrazones are hydrolysed to INH in the test procedure and the interference due to INH itself is small. The hydrazones could also be converted to INH by prior acid treatment of the urine, and the direct method then employed.

The presence of INAG in urine also leads to over-estimation of the Ac. INH content. However, the proportion of INAG that is estimated as Ac. INH is considerably less with the extraction method (4 per cent) than with the direct method (13 per cent) probably because of the incomplete extraction of the INAG or because of the hydrolysis of INAG to INA and glycine, neither of which affect the estimation of AC. INH.

It may be concluded that the extraction method is more specific than the direct method for the estimation of Ac. INH in urine. However, it involves an extra step—namely, obtaining the acid extract. In practice, it is not uncommon to estimate the content of both Ac. INH and INH for the same urine specimen (for instance, when determining the isoniazid inactivation rate from urinary excretions), and to obtain an acid extract for the estimation of INH. In such situations, the extraction method would obviously involve no extra labour.

Two other methods for the direct estimation of Ac. INH in urine, which can be employed even in the presence of glucose, have been described by Ellard *et al.* (1972). One is a fluorimetric method which is highly sensitive (0.2 µg./ml.) but slightly complicated, and requires special equipment. The other is a less sensitive (1 µg./ml.) but simpler calorimetric method, which involves the coupling of the hydrazine side chain with p-dimethylaminobenzaldehyde and suffers an interference of 43 per cent from DAH. The extraction method described in this paper depends on the cleavage of the pyridine ring, and consequently suffers no interference from DAH. It involves fewer steps than the calorimetric method of Ellard *et al.*, (1972) but is less sensitive (2 µg./ml). Both methods, however, are adequate for purposes of phenotyping patients as rapid or slow inactivators of isoniazid.

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